Final Scientific Report

Cover Page

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Project Title: Targeting of Strigolcatones associated pathways for conferring Orobanche resistant traits in Tomato and Medicago

Investigators **Institutions**

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Final Scientific Report

Keywords:

Parasitic plants

Arbuscular Mycorrhiza Fungi

Colonization

Root organ culture

Abbreviations commonly

AMF- Arbuscular mycorrhiza fungi

ROC- Root organ culture

P- Phosphorus

ORT- Orobanche resistance trait(s)

Budget: IS: $ 47,300 US: $ 53,500 Total: $ 100,800

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Signature	Signature
Principal Investigator	Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation				
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)	1			1

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description Cooperation: The project was carried by the Israeli and the US labs. The US lab carried all Medicago experiments, whereas the Israeli lab conducted all tomato experiments. Moreover, the TC 127050 gene and the relevant constructs were prepared by the US lab, and were transferred to the Israeli lab, for tomato transformation. Results and conclusion were shared between the labs. Also, during the

project the scientist met twice, in the US and Israel and discussed different aspects of the current project.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Final Scientific Report

Final Scientific Report (one copy by e-mail to lea@bard-isus.com **and four hard copies):**

Abstract

This proposal is focused on examination of two plant interactions: parasitic with Orobanche, and symbiosis with arbuscular mycorrhiza fungi (AMF), and the involvement of a newly define plant hormones, strigolactones (SLs), in these plant interactions. In addition to strigolactones role in regulation of above-ground plant architecture, they are also known to be secreted from roots, and to be a signal for seed germination of the parasitic plants *Orobanche.* Moreover, secreted strigolactones were recognized as inducers of AMF hyphae branching. The present work was aimed at Generation of RNAi mutants of both tomato and Medicago, targeting multiple genes that may be involved in strigolactone production, carotenoid biosynthesis pathway, Pi signaling or other metabolic pathways, and hence affect AMF colonization and/or Orobanche resistance. Following the newly formed and existing RNAi mutants were examined for AMF colonization and Orobanche resistance. At the first phase of this project Orobanche seed germination assays and AMF colonization were examined in intact plants. These assays were shown to be effective and resulted with enhancement of Orobanche seed germination and AMF colonization in WT tomato plants, whereas roots of strigolactones impaired lines did not result with Orobanche seed germination and mycorrhiza colonization. Unexpectedly, root organ cultures (ROC) that were produced from the same wild type (WT) and mutant lines did not induce the Orobanche seed germination and AMF hyphal branching. This implies that under in vitro conditions ROC cultures are missing an important component for induction of Orobanche seed germination and AMF hyphal branching. In another line of experiments we have tested transgenic lines of *Medicago truncatula* for AMF huyphal branching and Orobanche seed germination assays. These lines included lines silenced for a GRAS transcription factor (RNAi 1845), an NBS-LRR type resistance gene (RNAi 1847), a kinase (RNAi 2403) and a protein of unknown function (RNAi 2417). In all cases, five independent transgenic root lines showed altered AMF phenotypes with reduced or aberrant colonization patterns. Following, we transformed tomato plants with the *M. truncatula* TC 127050 Phosphoinositide kinase RNAi construct. Transgenic lines that contained GUS constructs were used as control. All transgenic lines showed reduced level of Orobanche seed germination, masking any strigoalctones-specific effect. The research demonstrated that SLs production may not be examined in ROC –based bioassays. It was shown by the 3 independent assays employed in this project that none of the recognized characters of SLs may be reflected in these bioassays. However, when the whole plant root exudates were examined, SLs activity in root exudates was demonstrated. Hence, it can be concluded that the presence of an intact shoot, and possibly, shoot factors, may be necessary for production of SLs in roots. Another point of interest that rises from these results is that the presence of SLs is not necessary for AMF completion of life cycle. Hence, it may be concluded that SLs are important for AMF hyphal branching, before symbiosis, but not essential for AMF colonization and life cycle completion under ROC system conditions.

Scientific Background and relevance to agriculture

Orobanche, commonly known as broomrape is a dicotyledonous achlorophyllous holoparasitic flowering plant (Family-Orobanchaceae: Order-Lamiales). It causes heavy economical losses in legumes, crucifers, tomato, sunflower, tobacco and many other crops in different parts of the world (Musselman, 1980; Joel, 2000). Broomrapes cause severe losses in crops even before the infection is diagnosed, and this makes it extremely difficult to control. Like other parasitic weeds, they develop a strong sink, which allows them to remove water, minerals, and photosynthates from the host crop. Thus, infection by parasitic weeds reduces the ability of the hosts to grow and yield.

O. aegyptiaca (and its close relative *O. ramosa*) and *O. crenata* are the most devastating Orobanche species that are responsible for most of the damage caused to vegetables and legume crops worldwide (Joel et al., 2007). ***Orobanche crenata*** **Forsk.** is an important pest in grain and forage legumes including ***Medicago truncatula***, devastating legumes that serve as an important source of protein in many Middle Eastern societies (Parker and Riches, 1993). ***Orobanche aegyptiaca*** **Pers.**, closely related to *O. ramosa*, is an important pest of many crops in Mediterranean countries in the Middle East and Africa, extending eastward to central Asia, India, China, and southern Russia. *Orobanche aegyptiaca* attacks crop plants belonging to various families, including Asteraceae, Brassicaceae, Cucurbitaceae, Fabaceae, and Solanaceae; including **tomato** (Parker and Riches 1993). Infestation of tomato and potato fields by *O. aegyptiaca* is especially dangerous, as these crops prove highly susceptible to *Orobanche* infection. Attempts to resume growing of a susceptible crop on infested land are liable to result with immediate re-infestation; extreme cases were documented in Israel, where *O. aegyptiaca* seeds survived in soil for more than 40 years (Joel et al., 2007).

Many different strategies are available for *Orobanche* management (Rispail et al., 2007; Joel et al., 2007), for example, sanitation and hand weeding (Ransom,2000), using selective herbicides (Hershenhorn et al.., 1998; Kleifeld et at., 1998), biological control (Sauerborn et al., 2007), soil treatments with fumigants and solar heating (Jacobsohn et al., 1980; Keleifeld et al., 1998) and trap crops (Sauerborn et al., 2007),

but most of the management strategies failed to provide a satisfactory control of the damages by this obligate parasitic weed (Joel et al., 2007).

Breeding for resistance is still one of the most effective, feasible and environment friendly management strategies against this weed (Rubiales et al., 2003). Efforts were done in many crops like chickpea (Rubiales et al., 2003), faba bean (Nassib et al., 1982; Roman et al., 2002; Rojas-Molina et al., 2006), pea (Valderrama et al., 2004) and sunflower (Perez-Vich et al., 2004) to develop molecular markers for *Orobanche* resistance, and breed crop varieties resistant to various species and races of *Orobanche*. Because of emergence of numerous resistant breaking races of *Orobanche* (Fernandez-Martinez et al., 2000), breeding a complete broomrape resistant variety is still to be achieved.

Arbuscular mycorrhizal fungi (AMF) are soil microorganisms that establish mutual symbiosis with the majority of higher plants. This symbiosis interaction is highly beneficial and substantially promotes plant growth and development. This association benefits plants by enabling them to grow and reproduce in relatively harsh mineral-stress environments. In return, the fungi obtain their carbon source from the host plant, in the form of plant photosynthates. This whole process of bidirectional nutrient exchange between plant and fungus is tightly linked and highly dependent upon environmental and biological variables. Interestingly, as described below, AM-symbiosis is utilizing, for establishment, the same signals that are responsible for Orobanche seed germination (which is the first stage of parasitism). Hence, it is most important to preserve the AM beneficial association, in accordance with the efforts to abolish the Orobanche parasitic association.

In the current proposal we would like examine the possibility to develop new means for the control of the parasite by reducing the capacity of plant crops to induce Orobanche seed germination, by suppressing the release (production or secretion) of the main seed germination signal – the strigolactones, from their roots to the rhizosphere. Yet, these efforts have will be done in accordance with the need to preserve the AM symbiosis. For that purpose we aim at extending our understanding of the pathways that are associated with strigolactones biosynthesis and release, to eventually be able to increase resistance to Orobanche while maintaining the ability of the plant to interact with AM fungi.

Key steps in the life cycle of Orobanche and arbuscular mycorrhiza fungi (AMF)

This proposal is focused on examination of two plant interactions: parasitic with Orobanche, and symbiotic with AMF. The parasite Oronbache produces thousands of dust-like seeds (0.2-0.3 mm) which are rapidly disseminated (Parker & Riches, 1993). The seeds germinate only in response to stimulants secreted by plant roots, which is provided after a conditioning period of a few days under moist conditions and suitable temperatures (Parker & Riches, 1993). The germinating seed produces a radicle that forms a haustorium when it comes in contact with the root and penetrates the root tissue, establishing a connection with the host vascular system (Joel et al., 2007; Parker & Riches, 1993). At maturity, the parasite develops a flowering shoot(s) that emerges above soil near host plants and sets seeds. The number of seeds produced by *O. aegyptiaca* plant can exceed 200,000 to half a million (Joel et al. 1995). The minute seeds may easily be transferred from one field to another by cultivation, and also by water, wind, animals, and by vehicles and farming machines.

Most AMF species form spores in the soil. The spores are capable of germinating in the absence of host-derived signals. The hyphae emerging from spores can grow to some extent but are unable to produce extensive mycelia and complete their life cycle without establishing a functional symbiosis with a host plant. There is increasing evidence that the fungus and plant recognize each other long before the appearance of the first colonization structures on the root epidermis. After hyphal brunching and following signaling exchange between the host root and fungus, a physical contact is formed: an appressorium is generated by the fungus following hyphal contact with the root, and epidermal penetration is formed in a cell-layer-controlled fashion (Genre et al. 2005; Siciliano et al. 2007). About 4 hr after appressorium formation, various cellular events are induced leading to a formation of two distinct intra-cellular starches, arbuscules and vesicules. After host colonization, the fungal mycelium grows out of the root exploring the soil in search of mineral nutrients, and it can also colonize other susceptible roots. Concomitantly, the fungus produces an extra-radical mycelium from which spores are eventually formed (Smith and Read 1997).

Strigolactones as a new compound of interest

Excitingly, in the last few years, it became clear that strigolactones may be considered as a new group of plant hormones. They are suggested to have a pivotal role in

regulation of above-ground plant architecture (Umehara et al 2008,) and inhibition of shoot branching (Gomez-Roldan et al 2008).

Strigolactones are also known to be secreted from roots, and to be a signal for seed germination of the parasitic plants *Orobanche* **(**Akiyama *et al* 2005). The seeds of *Orobanche* will only germinate after induction by a chemical signal exuded from the roots of their host and some non-host plants (Joel *et al* 2006). A strigol-like compound was isolated from root exudates of red clover and was shown to be a germination stimulant of *Orobanche minor* (Yokota *et al* 1998). Moreover, secreted strigolactones were recognized as inducers of AMF hyphae branching. Buee *et al* (2000) partially purified lipophilic substances from root exudates that promoted hyphal branching of AMF symbiotic mycelium; these compounds were identified as strigolactones (Matusova *et al* 2005).

The presence and role of the strigolactones support the notion that the plant-symbiotic interaction with AMF or plant –parasitic interaction with the parasitic plant *Orobanche*, share common biochemical signaling pathways, especially during the early stages of association, while the association is established on plant roots. Hyphal branching of AMF has been suggested as one of the events in host root recognition that precedes successful root colonization (Nagahashi and Douds, 2000). Host roots exudates were reported to induce branching of AMF hyphae (Akiyama *et al*., 2005). Also, in the AMF *Glomus intraradices* and *Glomus claroideum*, spore germination was also stimulated in the presence of strigolactones (Besserer *et al*., 2006). Application of GR24 (a synthetic strigolactone analogue) resulted in profuse branching of the AMF hyphae independent of the presence or absence of host roots (Gomez-Roldan *et al*., 2008).

The biosynthetic pathway of strigolactones in plants is not fully understood. Strigolactones were described as sesquiterpene lactones (Yokota *et al* 1998), however, their structures are also similar to higher terpenoids/isoprenoids (Bouwmeester *et al* 2007). Strigolactones are suggested to be derived from carotenoid cleavage products (Umehara et al., 2008; Matusova et al 2005; Lo´pez-Ra´ez et al., 2008 Naik *et al* 2003). Strigolactones are present in root exudates in extremely low concentrations (Akiyama and Hyashi 2006). They are unstable and have been detected in root exudates of host and non-host plants for parasitic plants (discussed below;

Steinkellner *et al* 2007). Presently, strigolactone molecules from tomato have partially identified (López-Ráez et al, 2008).

Notably, recent studies indicated that strigolactone concentrations in root exudates increase in response to phosphorous (P) deficiency and also in response to nitrogen deficiency (Yoneyama et al., 2007; Lopez-Raez et al., 2008). This is likely to stimulate both the interaction with AM fungi and the susceptibility to infection with Orobanche. However, currently the exact signaling pathways that mediate this regulation are still unknown.

Involvement of plant phosphorus levels

The plant P status has a regulatory effect on the AM symbiosis and fungal colonization of the root system is reduced when plants are grown in high-P conditions (Koide and Li, 1990; Koide, 1991). Current data suggest that plant P status affects both the initial colonization events, and the fungal development within the cortex. Split root experiments indicate that growth in high P conditions lead to a reduction in infection events (Menge et al., 1978; Thomson et al., 1991). Interestingly, strigolactone availability in the rhizosphere is also reduced in the presence of high P levels. Thus, it is tempting to suggest that the suppression of P on AMF-plant association may be mediated via strigolactones. Currently, it is not clear how this P effect is mediated and it is unknown if strigolactone plays any role during the later stages of the AMF symbiosis.

II. Concise outline of specific, feasible research objectives.

Research goal:

Specific objectives:

1. Generation of RNAi mutants of both tomato and Medicago, targeting multiple genes that may be involved in strigolactone production, carotenoid biosynthesis pathway, Pi signaling or other metabolic pathways, and hence affect AMF colonization and/or Orobanche resistance.
2. Screening of newly formed (Objective 1) and existing (Preliminary results) RNAi mutants for AMF colonization and Orobanche resistance.

Achievements:

1. Determination of the effect of growing root in culture on ability to induce Orobanche germination

a. Effect was examined for root growing in culture, for both "natural", i.e., induced and propagated without Agrobacterium, and those induced by *Agrobacterium rhizogenes* transformation. First, to establish a way for determination of root exudates effect on Orobanche germination, we have examined the effect of roots of plants grown in different growth media on their ability to induce orobanche seed germination. For this purpose we have employed wild type (WT; M82) as well as Sl-ORT1, which is a mutant line flawed in strigolactones (SLs) production. The collected WT root exudates of the young seedlings (4 weeks old) were able to induce orbanche seed germination under all examined growth media, including growth in vermiculite and perlite, irrigated with double distilled or tap water (Fig 1). In all cases, Sl-ORT1 plant mutants were not able to induce orobanche seed germination. It important to note that the positive control GR24, a synthetic SLs with a biological activity, was able to induce Orobanche seed germination at a rate of 89% of the seeds (Fig 1).

2. Determination of the effect of growing root in culture on ability to induce AMF hyphal branching

However, once hairy root cultures induced by *A. rhizogenes* of M82 were examined for orobanche seed germination, no seed germination was recorded. Moreover, the M82 root exudates ability to induce orobanche seed germination was even reduced than that of Sl- ORT1. It is important to note that the positive control, i.e., GR24, was able to induced Orobanche germination also in these experimental conditions (Fig 2).
Next, we thought that *A. rhizogenes* transformed roots exudates might be altered in aspects that prevented M82 culture to induce Orobanche seed germination. Hence, we have examined natural root cultures that have been grown under lab conditions as root culture for few generations had been tested for the effect of root exudates on Orobanche seed germination. The results suggested that there is no significant difference between M82 root cultures to ORT1 root culture, in terms of Orobanche

seed germination. The germination induction capacity was found to be relatively low per g of FW root

As a next step, we tried to modify the root culture growth conditions. Neither changing the vitamin content, or culture aeration and volume did not change the results (data not shown).

As detailed above, strigolactones are known also as inducers of mycorrhiza hyphal branching. To farther examine the ability of the root cultures exudates to induce SLs-associated processes, we have examined their effect on AMF hyphal branching.

Following, we have examined the transformed culture ability to support mycorrhizal hyphal branching. Root culture exudates were not able to induce hyphal branching in germinated *Glomus intraradices* spores. GR24, the positive control, induced hyphal branches significantly, relative to water.

3. Screening of existing RNAi mutants for AMF colonization and Orobanche resistance

Several transgenic root lines of *Medicago truncatula* were generated in the course of the project. We tested the AMF and Orobanche seed germination assay phenotypes of *M. truncatula* roots silenced for a GRAS transcription factor (RNAi 1845), an NBS-LRR type resistance gene (RNAi 1847), a kinase (RNAi 2403) and a protein of unknown function (RNAi 2417). In all cases, five independent transgenic root lines showed altered AMF phenotypes with reduced or aberrant colonization patterns. Additionally, several transgenic lines of tomato were generated in the course of the project. We transformed tomato plants with the *M. truncatula* TC 127050 Phosphoinositide kinase RNAi construct (RNAi 2403). Medicago TC 127050 encodes aphosphoinositide kinase. The gene was BLASTX in the NCBI database and a high score result was obtain for 3-phosphoinositide-dependent protein kinase-1 Lycopersicon esculentum (accession no. AAW38936.1). TBLASTN screening resulted in the gene ID: 544184 Pdk1 | 3-phosphoinositide-dependent protein kinase-1[Solanum lycopersicum]. The target Medicago sequence was aligned with the tomato gene sequence and 81% similarity was found in a 157bp fragment. Therefore, this Medicago construct was chosen for making tomato transgenic plants. From the

suggested list of Medicago constructs, no other RNAi constructs matched the tomato sequences sufficiently well to enable expression silencing.

We have generated 18 different transformed lines. These lines were raised under liquid conditions and root exudates from each of the lines were collected and their ability to induce Orobanche germination was examined. GUS expressing lines were raised and served as positive controls for seed germination. The results suggested that M82 induced about 5% of seed germination, whereas GR24 induced about 60% of germination. Most of the transgenic lines induced higher levels of germination than M82, however, the various GUS lines were also with higher levels of seed germination than M82 (Fig 5).

Strigolactones are known also as inducers of mycorrhiza hyphal branching, which might be important for successful AMF colonization on host root. To farther examine the ability of the root cultures exudates to induce SLs-associated processes, we have examined their effect on AMF life cycle.

Using the root organ culture assay, the number of AMF spores was determined, as a measure for their ability to complete life cycle. M82 root cultures supported a higher number of AMF spore generation than Sl-ORT1 (the deficient SL mutant) root culture (Fig 6). Moreover, all lines transformed with TC 127050, showed a high number of AMF spore production than M82. However, this was true also for the control of GUS transformed lines, suggesting that this phenomenon is not directly related to the transformed gene (Fig 6).

In Fig 7 an example for root culture (ROC) plates for in vitro assay of AMF spore production.

Since we could not obtain a clear relation between the transformed gene and SLs production in Tomato, we have examined the effect of its gene expression silencing on SLs production in *M. truncatula*.

Root exudates have been collected from each line and were used for the orobanche seed germination assay. As shown in Fig 8, only one transformed line showed reduction in induction of orobanche seed germination in comparison to M82. Hence, also in *M. truncatula*, no clear effect of TC 127050 silencing was demonstrated on SLs production.

Significance of main scientific achievements or innovations

The research demonstrated that SLs production may not be examined in ROC –based bioassays. It was shown by the 3 independent assays employed in this project that none of the recognized characters of SLs may be reflected in these bioassays.

However, when the whole plant root exudates were examined, SLs activity in root exudates was demonstrated. Hence, it can be concluded that the presence of an intact shoot, and possibly, shoot factors, may be necessary for production of SLs in roots.

Another point of interest that rises from these results is that the presence of SLs is not necessary for AMF completion of life cycle. Hence, it may be concluded that SLs are important for AMF hyphal branching, before symbiosis, but not essential for AMF colonization and life cycle completion under ROC system conditions.

Details of cooperation

The project was carried by the Israeli and the US labs. The US lab carried all Medicago experiments, whereas the Israeli lab conducted all tomato experiments. Moreover, the TC 127050 gene and the relevant constructs were prepared by the US lab, and were transferred to the Israeli lab, for tomato transformation. Results and conclusion were shared between the labs. Also, during the project the scientist met twice, in the US and Israel and discussed different aspects of the current project.

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Appendix:

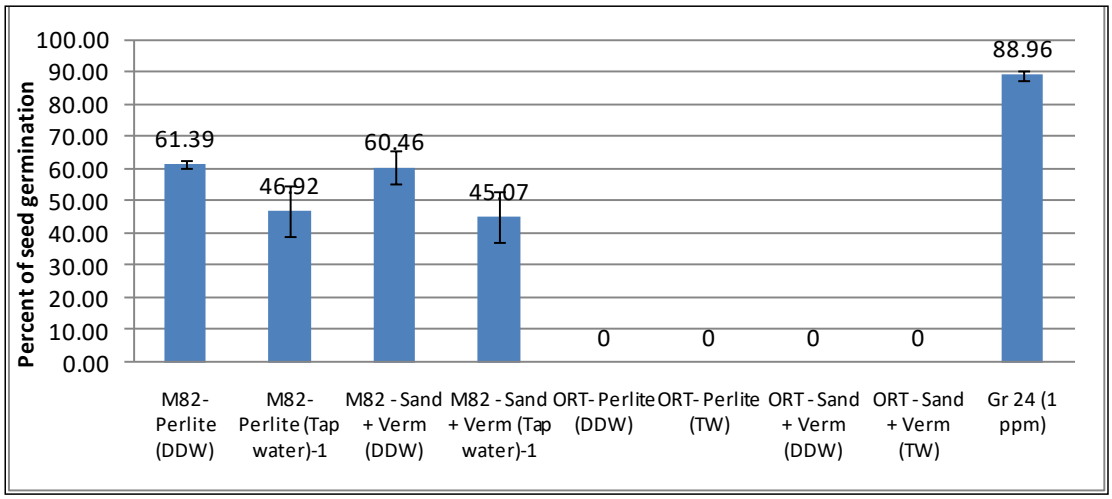

Fig 1: the effect of root exudates of greenhouse grown tomato plants on orobanche seed germination.

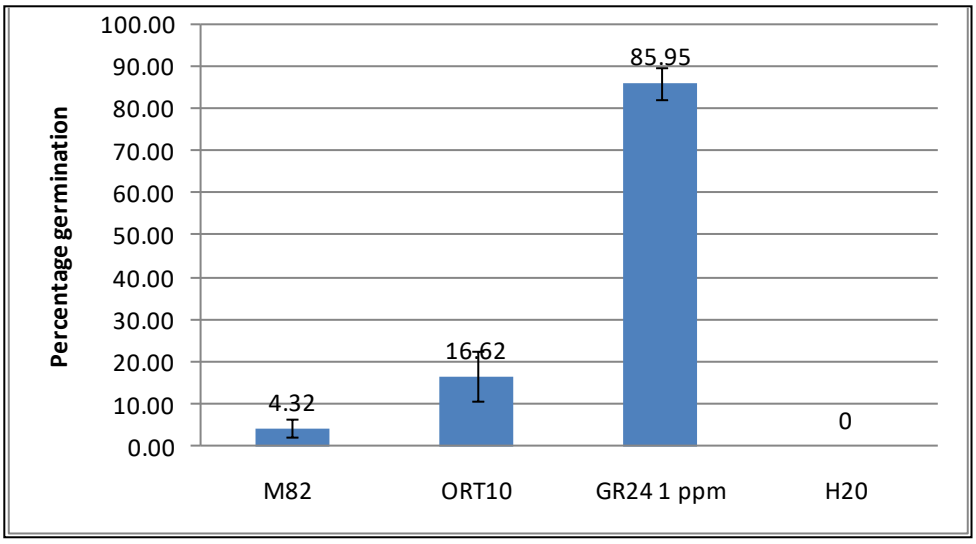

Fig 2: the effect of root exudates of hairy root cultures induced by *Agrobacterium rhizogenes* on orobanche seed germination.

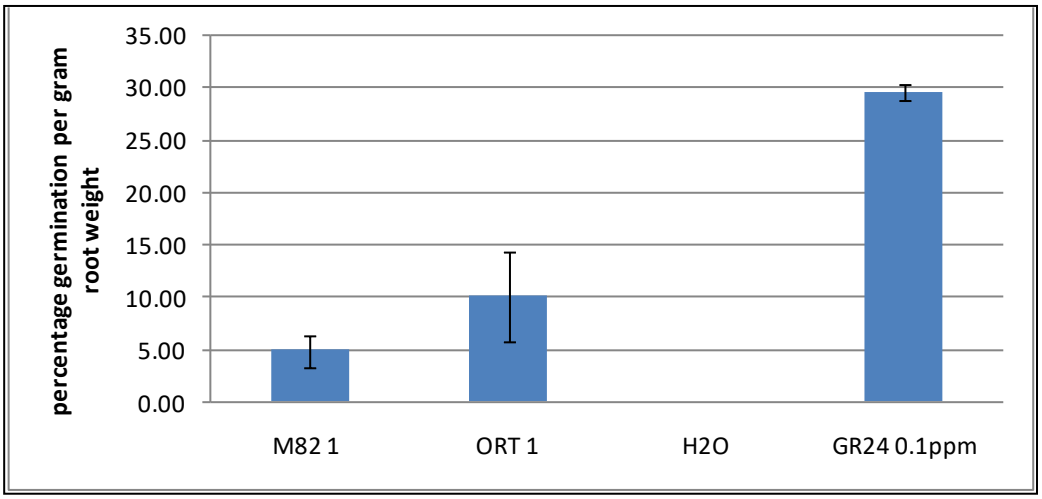

Fig 3: the effect of root exudates of non-transformed root cultures on orobanche seed germination.

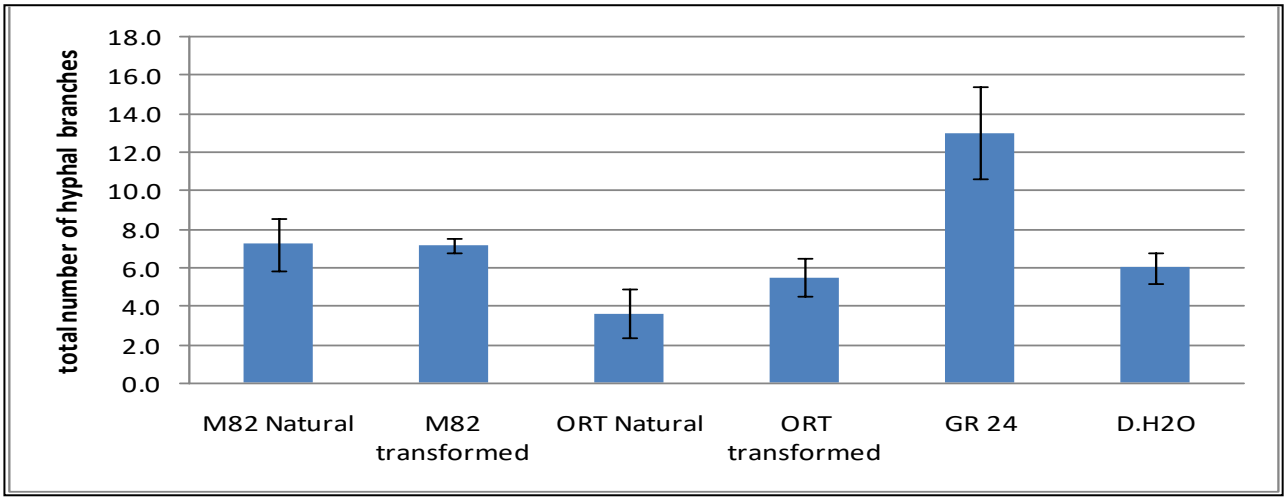

Fig 4: the effect of root exudates of both non-transformed (natural) and transformed root cultures on number of hyphal branches of AMF.

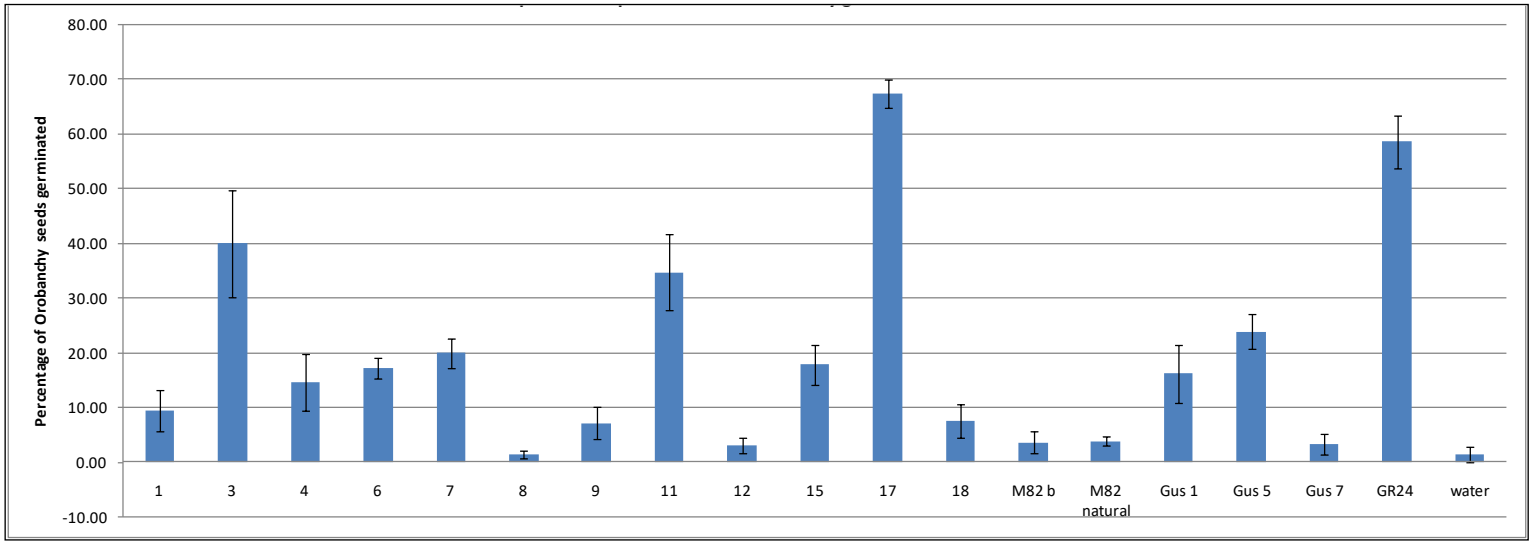

Fig 5: The effect of root exudates of TC 127050 and GUS transformed root cultures on orobanche seed germination.

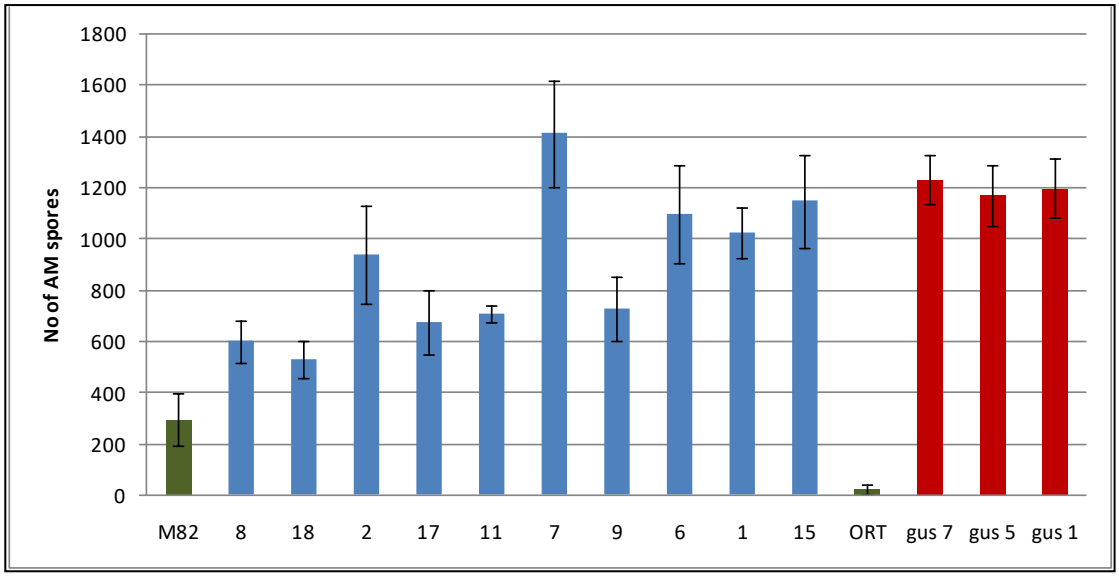

Fig 6: The effect of different root cultures to support AMF spore production under ROC growth conditions.

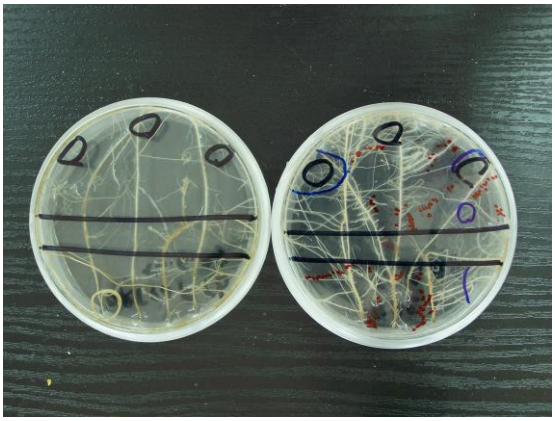

Fig 7: an example for root culture (ROC) plates for in vitro assay of AMF spore production.

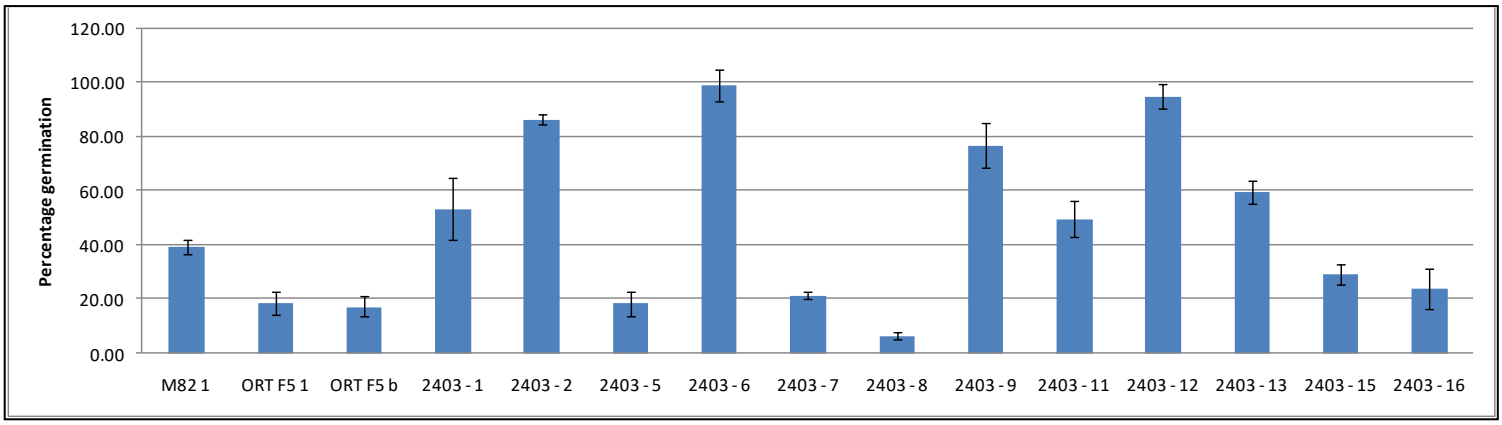

Fig 8: The effect of root exudates of TC 127050 and GUS transformed root cultures of *M. truncatula* on orobanche seed germination.